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Automated visual choice discrimination learning in zebrafish (*Danio rerio*)

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Training experimental animals to discriminate between different visual stimuli has been an important tool in cognitive neuroscience as well as in vision research for many decades. Current methods used for visual choice discrimination training of zebrafish require human observers for response tracking, stimulus presentation and reward delivery and, consequently, are very labor intensive and possibly experimenter biased. By combining video tracking of fish positions, stimulus presentation on computer monitors and food delivery by computer-controlled electromagnetic valves, we developed a method that allows for a fully automated training of multiple adult zebrafish to arbitrary visual stimuli in parallel. The standardized training procedure facilitates the comparison of results across different experiments and laboratories and contributes to the usability of zebrafish as vertebrate model organisms in behavioral brain research and vision research.

Keywords: Zebrafish; visual behavior; learning; memory; automated behavioral testing.

1. Introduction

Over the past decades, the zebrafish (*Danio rerio*) became one of the favorite model organisms in developmental biology, embryology, toxicology as well as pharmacology (Barinaga, 1990; Barros *et al.*, 2008; Vascotto *et al.*, 1997). In the field of behavior and behavioral neuroscience, however, it so far has received relatively little attention. With the nearly completed whole genome sequencing project by the Sanger institute and the rapidly growing genetic toolkit, which nowadays also allows for targeted mutagenesis (Doyon *et al.*, 2008; Meng *et al.*, 2008; Moens *et al.*, 2008; Wienholds *et al.*, 2003), more and more scientists from various fields start to recognize the potential of this small teleost. The advantages of a highly prolific vertebrate species showing a rich repertoire of behaviors (e.g., food searching behavior, shoaling behavior, aggressive and submissive behavior), in combination with the constantly improving genetic techniques open the field for promising behavioral studies.

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February 22, 2012 7:26:43pm WSPC/179-JIN 1250005 ISSN: 0219-6352 FA1

For vision research, zebrafish as diurnal animals are especially valuable because of their rapidly developing, cone-dominated retinas. (Fadool & Dowling, 2008) With the optokinetic (Rinner *et al.*, 2005; Huang & Neuhauss, 2008), optomotor (Orger & Baier, 2005; Orger *et al.*, 2000) and visual motor (Emran & Dowling, 2008) response assays, scientists have an elaborated toolbox at hand, which allows them to behaviorally screen for mutations and characterize the visual system of larval fish in an automated fashion. For adult fish, however, the situation is less satisfying. While in the past, the need for behavioral tests to probe the visual system of adult fish has been marginal, since most mutations detected in forward-genetic screens are lethal within the first days of development, the possibility to knock down specific genes of interest through zinc-finger nucleases (Doyon *et al.*, 2008; Meng *et al.*, 2008) or TILLING (targeted induced local lesions in genomes) (Moens *et al.*, 2008; Wienholds *et al.*, 2003) will certainly lead to a tremendous increase in the number of potentially interesting mutant strains viable through adulthood and showing late-onset visual defects. The investigation of such genetic defects is of outstanding interest, since most hereditary diseases leading to blindness in humans, including age-related macular degeneration and retinitis pigmentosa, develop with age (Chen *et al.*, 2010; Hartong *et al.*, 2006). With the increasing life-expectancy in developed and emerging nations, these diseases can be expected to pose an ever increasing challenge to our health care systems. Although the larval optokinetic response assay was recently adapted to adult fish (Mueller & Neuhauss, 2010), and optomotor and visual motor response assays are feasible as well, for a thorough investigation of visual function and, especially, of color vision, additional assays are needed.

On the other hand, the zebrafish also lends itself to study processes of learning and memory formation, their molecular basis and the effects of genetic or pharmacological perturbation on neural development. Different approaches have been employed to study learning and memory in zebrafish, e.g., by using shuttle-boxes (Pather & Gerlai, 2009; Pradel *et al.*, 1999, 2000; Williams *et al.*, 2002; Yang *et al.*, 2003), T-mazes (Colwill *et al.*, 2005; Yu *et al.*, 2006), three compartment mazes (Arthur & Levin, 2001; Eddins *et al.*, 2009; Levin *et al.*, 2006, 2003; Levin & Chen, 2004), plus-mazes (Al-Imari & Gerlai, 2008; Sison & Gerlai, 2010) or conditioned place preference assays (Darland & Dowling, 2001; Lau *et al.*, 2006; Yu *et al.*, 2006).

A method which can be used to study visual function as well as learning processes and memory retention was recently developed (Bilotta *et al.*, 2005) and successfully used to determine the behavioral spectral sensitivity of adult zebrafish (Risner *et al.*, 2006). Similar methods have been used for many years to study, e.g., color contrast and color constancy in goldfish (Dorr & Neumeyer, 2000, 1997; Neumeyer *et al.*, 2002). However, learning in fish is comparatively slow, and, when done manually as in the aforementioned studies, very time consuming. At the same time, the presence of a human observer in the testing room might influence the learning success and impede comparison of results across different studies and laboratories.

February 22, 2012 7:26:44pm WSPC/179-JIN 1250005 ISSN: 0219-6352

For these reasons, we developed a fully automated operant conditioning setup, which allows appetitive training of zebrafish to arbitrary visual stimuli. Our apparatus can be used to train up to eight fish in parallel, and no operator needs to be present.

2. Material and methods

2.1. *Subjects*

For all experiments, we used adult (> 1 year) zebrafish of the wild-type strain WIK obtained through crossings in our own fish facility. Prior to experiments, fish were raised and maintained in a circulating water fish facility (Aqua Schwarz GmbH, Germany) at a temperature of 28°C under a constant light-dark regime (L:D = 14 h:10 h). They were daily fed with dried flake food in the mornings and living, freshly hatched brine shrimps in the evenings. Since zebrafish are highly social animals, they were separated 1 week in advance of an experiment in order to habituate them to isolation from the group. During this time, as well as throughout the experiments, they were kept in standard polystyrene single-tanks (L × W × H = 100 × 200 × 85 mm, Brac-Werke AG, Switzerland) under the same light regime and at the same temperature. Water was not circulating in these tanks, and the water was exchanged manually every second week. To ensure a strong motivation, experimental animals were food-deprived for 3 days prior to any experiments. Animal care and all experimental procedures were carried out in accordance with the European Communities Council Directive (86/609/EEC).

2.2. *Training apparatus*

In our current setup, up to eight fish can be trained in parallel. Eight compartments are formed by opaque grey PVC-walls (height = 85 mm) mounted on a translucent baseplate made of acrylic glass. In each of the eight compartments, one standard single-tank can be placed (Fig. 1). Each tank is illuminated from below with an array of 28 infrared-emitting diodes (λ_{peak} = 875 nm; M120, Kemo, Germany) mounted at a distance of 27 cm. The setup is filmed by an infrared-sensitive CCD-camera (Stingray F-046B, Allied Vision Technologies, Germany) placed 125 cm above the apparatus. The camera is equipped with a high-resolution lens (CF12,5HA-1/1,4, Fujinon, Japan) and an infrared-pass filter (FIL 093/49, Schneider-Kreuznach, Germany).

On both sides of the setup, a 22″ LCD-display (E220HD, BenQ Corporation, Taiwan) is mounted (Fig. 1). The displays are protected against water damage by a thin, transparent plastic foil (d = 0.02 mm; 7 611709 171217, Jumbo-Markt *AG*, Switzerland).

Custom-made software based on NI LabView 2009 and NI Vision Development Module 2009 (National Instruments, Austin, TX) is used to track the position of the fish, control stimulus presentation and food delivery. In short, the tracking

February 22, 2012 7:26:44pm WSPC/179-JIN 1250005 ISSN: 0219-6352

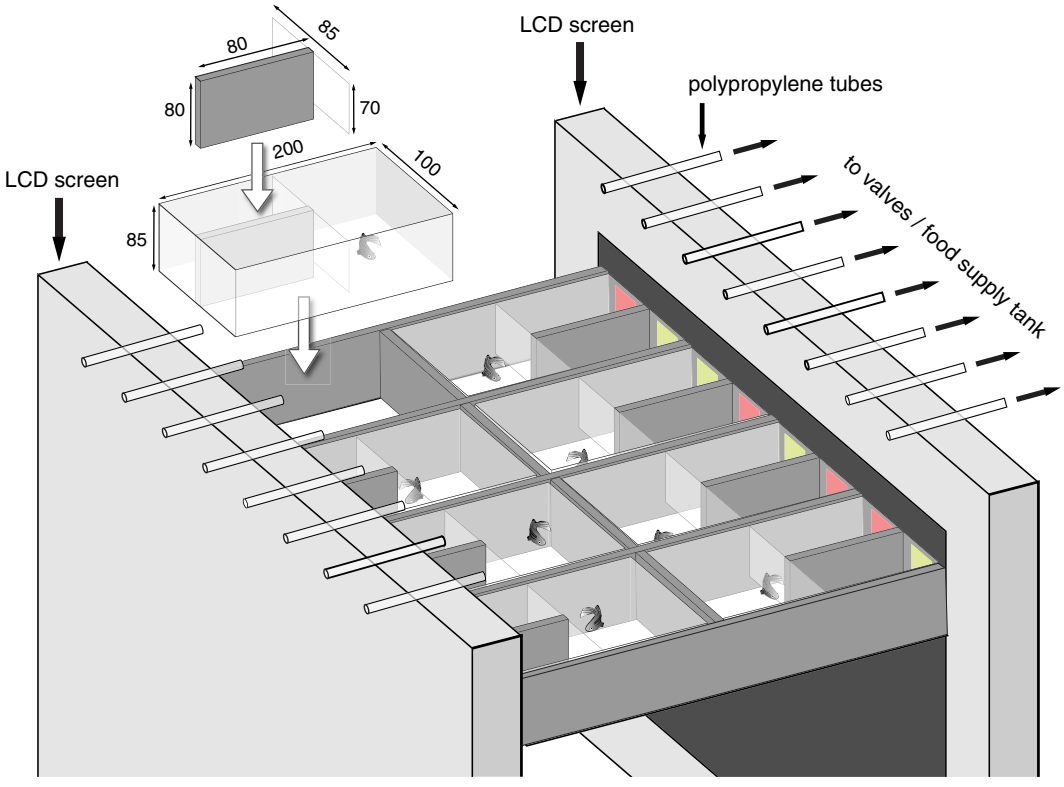

Fig. 1. Schematic drawing of the training apparatus in the configuration for training eight fish to discriminate between two different stimuli. Length measures are given in mm.

algorithm works as follows: Images from the camera are evaluated at 20 frames per second (fps). We use an adaptive background subtraction with a delay of 20 frames, i.e., from every frame, the frame taken 1 s before is subtracted. The resulting image (showing only the parts which moved within the last second) is binarized using a fixed threshold, and detected particles are filtered by size to reduce noise. We then use a point-to-nearest-neighbor algorithm to track every single fish, whereby the possible positions for each fish are confined to the space occupied by its tank. If no particle is detected within this space, the fish is assumed having not moved since the last frame. An additional threshold is set for the maximal distance a fish is allowed to move between to successive frames to avoid the track from accidentally jumping to a far-away (noise) particle if the actual fish was not detected, i.e., if the fish has not moved.

As food reward, we use living brine shrimps. Food delivery into each choice chamber is controlled by 16 electromagnetic valves (VDW11-5G-2-M5-H-Q, SMC Corporation, Japan), interconnected to two islands of eight valves each and attached on the backside of each monitor. Each valve is connected to a separate channel of one of two USB-controlled relay cards (USBREL8, QUANCOM Informationssysteme GmbH, Germany), allowing computer-controlled opening and

February 22, 2012 7:26:45pm WSPC/179-JIN 1250005 ISSN: 0219-6352 FA1

closing of each individual valve. The food-supply tank consists of a standard wash bottle turned upside-down with the bottom cut-off. To ensure survival of the brine shrimps for a whole training-day, and to keep the solution mixed, an air-pump (R301, Rena, PA) is used. The food-supply tank is mounted 30 cm above the apparatus and connected via a Y-junction to both valve islands using polypropylene tubes (ø = 7 mm). Outlets of the valves are connected to polypropylene tubes (ø = 6 mm) as well. The ends of the outlet tubes are attached to the displays top edges and overhang it by 2 cm, such that each ending is positioned above one choice chamber (Fig. 1).

2.3. *Training procedure*

To minimize stress induced by handling (i.e., netting) or change in water quality, fish are trained in their homing tanks. Two choice chambers are formed by introducing a T-shaped plastic divider, consisting of an opaque grey PVC-wall attached to a clear front-end made of acrylic glass (Figs. 1 and 2). The front-end is raised by 1 cm to allow the fish access to the two choice chambers separated by the grey PVC-wall. This T-shaped divider is introduced into each tank on the eve of each experimental session and removed immediately after a completed session. Each tank is then placed in one of the eight compartments, the light in the room is switched off and the operator leaves the room. After a completed session, the tanks are returned to a climate room with constant temperature and light regime, and the next set of tanks is equipped with T-shaped dividers and placed in the training apparatus. In the training apparatus, water was neither circulating nor was the temperature regulated.

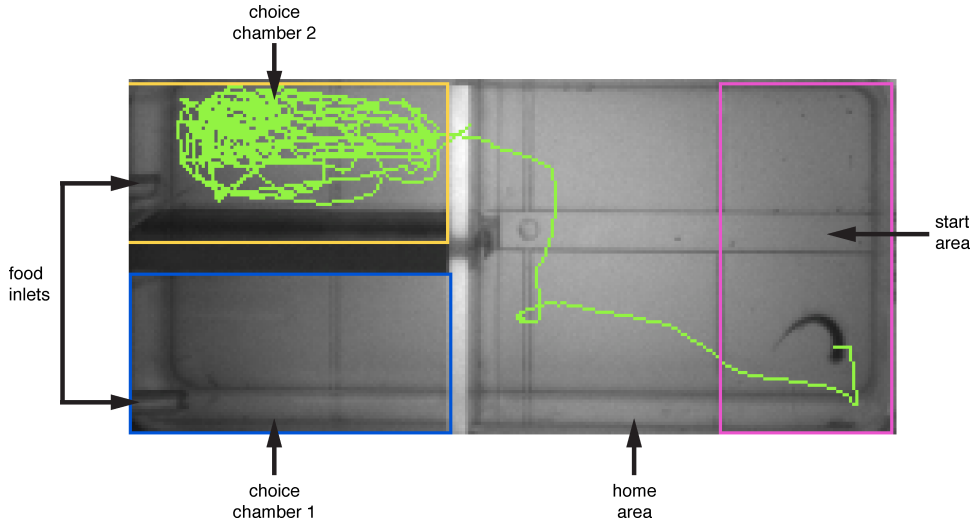

Fig. 2. Typical example of a trial where a fish chose the correct compartment: the fish staid in choice chamber 2 until all brine shrimps were consumed. After leaving the choice chamber, the fish has to return to the start area for the next trial to start.

February 22, 2012 7:27:18pm WSPC/179-JIN 1250005 ISSN: 0219-6352 FA1

2.3.1. *Initial tendency test*

In order to detect possible natural preferences for one or the other visual stimulus, an initial tendency test is performed in the beginning. Fish are habituated for 10 min to the training apparatus. During this time, no stimuli are presented on the screen. After 10 min, two different stimuli (differently colored squares) are randomly assigned to the two choice chambers of each tank and constantly presented for 20 min. During this time, the position of each fish is recorded and from the recorded positions, the time spent in each compartment is calculated.

2.3.2. *Pre-training*

Pre-training sessions lasting 30 min are performed one day after the initial tendency test. Again, fish are first habituated to the apparatus for 10 min. After 10 min, the two stimuli are randomly assigned to the two choice chambers and constantly presented for 20 min. Each time a fish enters the chamber where the correct stimulus is presented, a small amount of food (approximately 10 brine shrimp) is delivered to this chamber by opening the respective valve for 200 ms. To avoid overfeeding of a fish, no food will be delivered on successive entries during 30 s following a food delivery. One session of pre-training is performed on each of two consecutive days, whereby the position of the reinforced stimulus is not counterbalanced in these two sessions.

2.3.3. *Training*

The day after the second pre-training, the training sessions are initiated. Fish are again first habituated to the training apparatus for 10 min. After 10 min, the two stimuli are pseudo-randomly assigned to the two choice chambers (i.e., randomly, with the constraint that the correct stimulus is not allowed to be on the same side for more than three consecutive trials). If the fish enters the chamber where the correct stimulus is presented, a small amount of food is delivered (again consisting of approximately 10 living brine shrimps). The fish is given time *ad libitum* to consume the food reward. During this time, the stimuli are left on. Only after the fish has left the choice chamber, the stimulus is switched off. The fish has then to return to the back of the tank (start area, see Fig. 2) in order for the next trial to start. If the fish enters the chamber where the wrong (non-rewarded) stimulus is presented, both stimuli are immediately switched off, the fish has to wait for 30 s and return to the start area to start the next trial. If the fish does not enter any of the choice chambers during 90 s, both stimuli are switched off, the fish has to return to the start area and a new trial starts. One training session consists of 20 trials. After the last fish has finished the 20th trial, a notification email is sent to the operator, making it unnecessary to enter the training room during an ongoing session. Fish were trained on 5 days a week, per training-day, each fish received between two and four training sessions. The inter-session interval on the same day was at least 1 h.

If a fish did not complete a training session within about 1 h, the session was aborted and the results from this fish were excluded from analysis.

February 22, 2012 7:27:19pm WSPC/179-JIN 1250005 ISSN: 0219-6352 FA1

2.4. *Discrimination between multiple stimuli*

To train fish to discriminate between more than two stimuli, the baseplate with the eight compartments can be exchanged by another one with only one separating PVC wall in the middle. On each side of the wall facing one of the screens, one large single-tank can be placed ($L \times W \times H = 198 \times 295 \times 75$ mm, Brac-Werke *AG*, Switzerland). Large-single tanks can be divided into 6 choice chambers by introducing a rake consisting of five grey PVC-walls attached to a clear front-end made of acrylic glass. As in the case of two choice chambers, the front-end is lifted by 1 cm to allow the fish access into the choice chambers. Training procedure was the same as in the case of two choice chambers, except that the habituation time was decreased to 5 min and the number of trials per session increased to 30.

2.5. *Data analysis*

Our custom-made software automatically saves images of the track for every single trial. In addition, for each trial and each fish, a separate file containing the positional coordinates, momentary swimming speed and distance covered so far is written to disk. A summarizing spreadsheet indicates number and percentages of correct and wrong choices as well as of trials where no choice was made, mean latencies (time between onset of a stimulus and entering of a choice chamber) and total time used for completion of a session for each fish and session. In a more detailed spreadsheet, the correct and the chosen compartment, correct and chosen stimulus as well as the latency are given for every single trial. For statistical analysis and generation of graphs, R 2.9.2 (www.R-project.org) and PASW Statistics 18.0 (IBM Corp., NY) was used.

3. Results

3.1. *Discrimination learning of two stimuli*

In the first experiment, we used 16 adult zebrafish, which were trained to discriminate a blue (R,G,B = 0,0,128) from a green stimulus (R,G,B = 0,128,0). The fish did not show any initial tendency towards the one or the other color: during 20 min, they spent 204.8 ± 49.5 s (mean $\pm$ 1 S.E.M) in the compartment where the blue stimulus was presented and 208.5 ± 41.1 s in the compartment where the green stimulus was presented. Since there was no obvious preference, all fish were subsequently trained to the blue stimulus. During the two sessions of pre-training, the time spent in the compartment where the correct (blue) stimulus was presented increased from 169.8 ± 55.2 s to 468.7 ± 107.2 s. In the same time, the time spent in the compartment where the wrong (green) stimulus was presented decreased from 648.3 ± 91.0 s to 304.5 ± 90.6 s. In the following training, fish started slightly below chance level ($37.4 \pm 5.3\%$ correct choices), but quickly increased their performance, reaching $80.0 \pm 2.9\%$ of correct choices in session 30 (Fig. 3(a)). The increase of performance in the course of training is highly significant (repeated measures

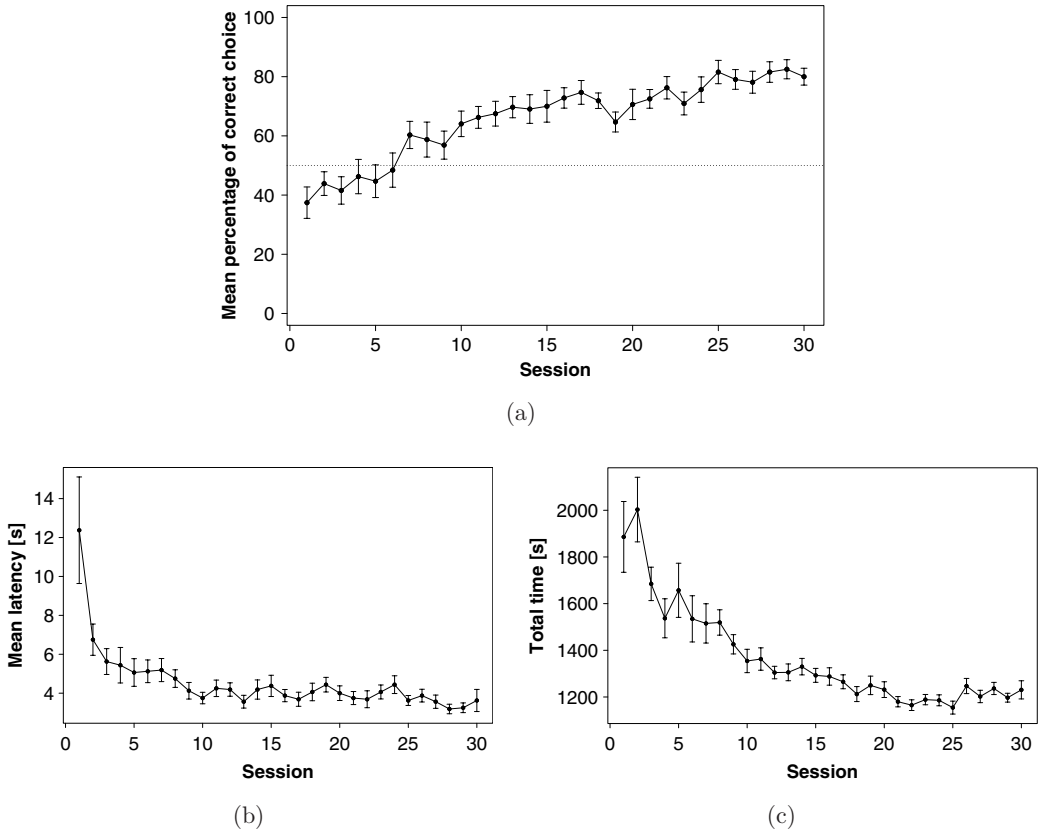

Fig. 3. Training of fish to discriminate a blue (R,G,B = 0,0,128) from a green (R,G,B = 0,128,0) stimulus. (a) Percentage of correct choice (performance), (b) latency, and (c) total time for completion of a session. The dotted line in (a) indicates chance level (50%). Values are means of 16 adult zebrafish ± 1 S.E.M.

ANOVA, $F_{1,11} = 431.37$, $p < 0.001$) and the performance in session 30 significantly higher than chance (one-sample t-test, $t_{15} = 10.53$, $p < 0.001$). In the same time, the mean latency (time between onset of stimuli and entering of a choice chamber) decreased from 12.4 ± 2.7 s to 3.6 ± 0.6 s (Fig. 3(b)), and the average total time for completion of a session from 1885.9 ± 151.7 s to 1230.6 ± 38.9 s (Fig. 3(c)). While in the first training session, 5 out of 16 fish did not always make a choice within the given 90 s (three fish once, one fish four and one five times, respectively), this never happened after the second training session.

3.2. *Discrimination of multiple stimuli*

Initially, seven fish were trained to discriminate between a blue (R,G,B = 0,0,128) and a green stimulus (R,G,B = 0,128,0) in the same way as above for 20 sessions (data not shown). One fish did not learn the task satisfyingly, most probably as the result of a leaking valve in one of the compartments, and was therefore not used for any further tests. The other six fish were transferred into large single tanks.

February 22, 2012 7:27:20pm WSPC/179-JIN 1250005 ISSN: 0219-6352 FA1

Subsequently, they were trained in the six choice chamber configuration to choose the blue stimulus, which was simultaneously presented with the green stimulus in all other compartments (data not shown). After seven sessions of training in this configuration, fish were further trained to choose the blue stimulus among a choice of different colors ranging from green to purple (R,G,B = 0,128,0/0,128,128/0,80,100/60,0,180/50,60,100). Although the average performance never fell below chance level (16.67%), this task seemed to be relatively difficult for the fish, and performance was only slowly increasing, reaching $58.2 \pm 7.1\%$ in session 164 (Fig. 4(a)). Overall, fish chose the blue stimulus most often with a relative choice frequency of 41.7%, followed by one of the purple stimuli with a frequency of 27.5% (Fig. 4(b)). The tuning to the blue stimulus clearly improved over the course of the training: In the first session, relative choice frequency of the blue stimulus was only 27.2%, even lower than for one of the purple ones (29.4%; Fig. 4(c)). In the last session, the respective choice frequencies were 58.3% (blue) and 26.7% (purple; Fig. 4(d)), which is significantly higher than expected by chance for the blue stimulus (one-sample t-test, $t_5 = 5.86$, $p = 0.002$).

4. Discussion

The apparatus presented in this paper allows for a fully automated, visually guided operant conditioning of up to eight fish in parallel. Since no human observer needs to be present in the training room, potential distractions or disturbances are minimized and large number of trials can be accomplished. In addition, the training procedure is standardized which facilitates comparison of results across different experiments or laboratories.

Training of fish in their home tanks renders netting of fish and transferring them into a training apparatus unnecessary, thereby significantly reducing stress for the experimental animals. As we noticed in prior experiments, this extremely improves learning success and diminishes habituation time needed in advance of every session, thereby decreasing total time needed for a session and increasing number of sessions feasible within a training day.

The use of LCD screens enables the presentation of arbitrary stimuli not confined to colored squares. In fact, we can use any desired images as stimuli and were, e.g., able to successfully train fish to discriminate between a very fine and a coarse black-and-white checkerboard pattern (data not shown). To use the setup for experiments concerning color vision, however, careful characterization and calibration of the displays by means of a spectroradiometer would be necessary.

The amount of food delivered per successful choice can be adjusted by changing the mounting height of the food supply tank, the density of brine shrimps in the stock solution as well as the time the valves open. The number of brine shrimps delivered by this method is not always exactly the same, but variability is relatively small (estimated to be smaller than four brine shrimps) and, importantly, is not biased towards one or the other choice chamber. The total amount of food fish

February 22, 2012 7:27:20pm WSPC/179-JIN 1250005 ISSN: 0219-6352

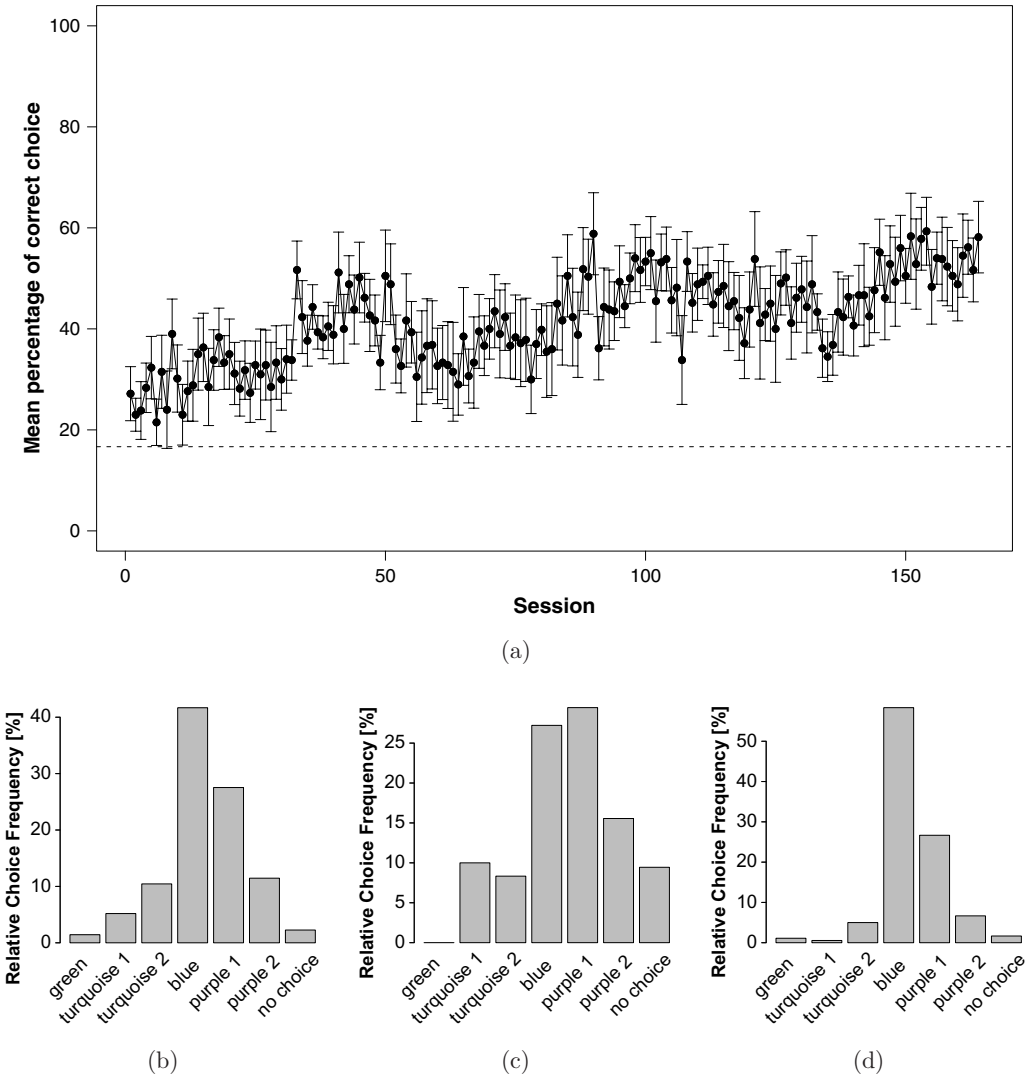

Fig. 4. Training of fish to discriminate a blue (R,G,B = 0,0,128) from five different stimuli ranging from green to purple (R,G,B = 0,128,0 (green)/0,128,128 (turquoise 1)/0,80,100 (turquoise 2)/60,0,180 (purple 1)/50,60,100 (purple 2)). (a) Percentage of correct choice (performance). The dotted line indicates chance level (16.67%). Values are means of 6 adult zebrafish ± 1 S.E.M. (b)−(d) Histograms of relative choice frequencies (b) over the whole training, (c) in the first session, and (d) in the last session.

received during the training was sufficient to keep them at good health without the need of additional feeding. At the same time, fish were still motivated to eat, even after four training sessions on the same day.

The data presented in this article show that using this apparatus, fish can be trained to discriminate between two differently colored squares quickly. Once they reach a performance of about 80% correct choices, performance stays relatively stable and fish also remember the task well after a short break of training, i.e., after weekends.

February 22, 2012 7:27:21pm WSPC/179-JIN 1250005 ISSN: 0219-6352 FA1

Fish refusing to exit a choice chamber or freezing in a corner might necessitate premature abortion of a session. Since zebrafish are very active swimmers, such long non-responding fish are in our experience quite rare, occurring in less than 10% of all tested fish and only during the first 5−10 sessions. However, such individual variation, if persistent in subsequent experiments, may lend itself to be a subject of investigation.

Discrimination learning of more than two stimuli can also successfully be achieved by our method, although quite a lot of training sessions are needed, and frequent relapses occur in this demanding task. A training to discriminate multiple colored stimuli as presented here may be useful to investigate more complex aspects of color vision, as for example color constancy.

As Robert Gerlai pointed out in a recent review article about high-throughput behavioral screens for zebrafish (Gerlai, 2010), behavioral test paradigms, and especially automated ones, still represent a bottleneck in zebrafish research. We believe that the apparatus presented here can significantly contribute to fill this gap and advance the investigation of not only brain function and memory, but also of the visual system of this promising vertebrate model organism.

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February 22, 2012 7:27:21pm WSPC/179-JIN 1250005 ISSN: 0219-6352 FA1

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